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Characterization of Human Monoclonal Antibodies Specific to the Hepatitis C Virus Glycoprotein E2 with *in Vitro* Binding Neutralization Properties

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Both linear and conformational determinants of hepatitis C virus (HCV) are believed to be involved in viral neutralization. After immortalization of B cells from HCV chronically infected patients with Epstein-Barr virus, we obtained two polyclonal lymphoblastoid cell lines (LCL) secreting human monoclonal antibodies (HMabs). One clone was derived from a patient infected with a genotype 4 isolate while the second was isolated from a genotype 1b-infected patient. Immunoprecipitation studies, Western blot, and immunofluorescence analysis, peptide scanning, and ELISA studies indicated that the HMabs (1) recognized conformation-dependent determinant(s), (2) were capable of recognizing genotype 1a and 1b derived antigens, and (3) were able to precipitate noncovalently associated E1E2 complexes believed to exist on the surface of virion particles. The HMab derived from the genotype 4-infected patient was in addition shown to neutralize the *in vitro* binding of recombinant E2 protein onto susceptible cells suggesting a potential for *in vivo* neutralization. These data indicate that anti-E2 antibodies directed at conserved conformational-dependent determinant(s) exist in chronic HCV infection.

INTRODUCTION

Hepatitis C virus (HCV) is the principal causative agent for non-A, non-B hepatitis. The prevalence of HCV infection in the blood donor population has been estimated to range from 0.4 to 2% (Choo *et al.,* 1989). Acute HCV infection leads, in more than 70% of the patients, to the development of chronic hepatitis, which can evolve toward cirrhosis and hepatocellular carcinoma (Saito *et al.,* 1990). HCV is an enveloped positive-stranded RNA virus which is classified in the Flaviviridae family (Francki *et al.,* 1991, Miller and Purcell, 1990). It contains a genome of about 9500 nts encoding a polyprotein of 3010–3033 amino acids. Processing of the polyprotein by host and viral proteases results in the production of structural and nonstructural (NS) proteins (Rice, 1996). Structural proteins include a nucleocapsid and two putative virion envelope glycoproteins E1 and E2 (Miyamura and Matsuura, 1993). Nonstructural proteins include NS2–NS5 antigens.

In some individuals, acute infection successfully resolves, indicating that HCV can be controlled by the host immune system. The mechanisms by which the host overcomes HCV infection remain unknown. Previous reports strongly suggest that humans and chimpanzees can generate virus-neutralizing antibodies (Choo *et al.,* 1994, Farci *et al.,* 1994, 1996, Shimizu *et al.,* 1994). Successful *in vivo* protection of chimpanzees from primary infection by an homologous HCV isolate has been achieved following immunization with recombinant E1 and E2 proteins (Choo *et al.,* 1994). In that study, only those chimpanzees showing high anti-E2 antibody titers were protected. While neutralizing antigenic domains were not identified, it was postulated that conformation of the immunogens was critical for the induction of neutralizing antibodies.

As there is to date no efficient *in vitro* replication system to grow the virus and develop neutralization assays, alternative assays to assess the biological function of anti-E1/E2 antibodies are being actively searched for. Prevention of viral attachment onto presumed susceptible cells has been described in preliminary studies (Shimizu *et al.,* 1994, Zibert *et al.,* 1995). More recently, an *''in vitro''* neutralization of binding (NOB) assay has been developed that is exploiting the specific binding of a highly purified E2 protein onto susceptible target cells (Rosa *et al.,* 1996). This assay allows the quantitative evaluation of NOB antibodies that are capable of neutralizing the binding of E2 onto such cells. Using this system, Rosa *et al.* (1996) have shown that only those

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TABLE 1

Characteristics of Patients and Derived Human Anti-E2 Monoclonal Antibodies

Patients	Genotype[a]	Viral load[b] Eq/ml $\times 10^5$	Histological diagnosis	HMAbs	Isotype[c]
1	4	5.2	Mild chronic hepatitis	503	IgG1λ
2	1b	21.8	Cirrhosis	108	IgG1λ

[a] Analysis were made with samples derived from the day of patients' EBV-PBMC transformation as well as from two different time points during a two year follow-up. Results at the different time points and between the different assays were concordant.

[b] Quantified in serum with the Quantiplex bDNA assay (Quantiplex HCV RNA Assay, Chiron Diagnostics, Emeryville).

[c] Supernatants from each clone were tested by IFA on CV-1 infected cells with the recombinant SVE2 virus and staining was revealed using specific secondary antibodies for human IgM, IgG, IgA, IgG1-4 subclasses.

chimpanzees immunized with E1 and E2 proteins that developed high anti-NOB titers were protected against challenged infection, suggesting that NOB activity could be an indication for *''in vivo''* neutralization of viral infection. In HIV infection, a similar model has recently shown that affinity of antibody binding to envelope glycoprotein oligomers was a good predictor for virus neutralization (Fouts *et al.,* 1997). Another way to assess the biological activity of anti-E1 and/or anti-E2 antibodies consists of testing the ability of such antibodies to recognize native structures believed to exist on the surface of virions. *In vitro* studies have shown that E1 and E2 interact to form noncovalently linked complexes (Ralston *et al.,* 1993; Deleersnyder *et al.,* 1997). Such complexes have been proposed to represent functional subunits of HCV virions (Ralston *et al.,* 1993; Dubuisson *et al.,* 1994; Dubuisson and Rice, 1996; Deleersnyder *et al.,* 1997).

In the present study, we have used a conformation-dependent antibody screening assay specific for the HCV E2 glycoprotein with the goal of generating human monoclonal antibody (HMAb)-producing cell lines. We successfully derived two HCV-producing clones from two chronically infected patients and analyzed the immune reactivity of the produced HMAbs in terms of epitope mapping, capacity to recognize noncovalently linked E1E2 complexes, and detection of NOB activity.

RESULTS

Patients' screening and generation of human monoclonal antibody producing B cells (LCLs)

Table 1 summarizes characteristics of the two patients and of the two lymphoid B-cell lines producing HMAbs, designated 503 and 108. Analysis of culture supernatants from the two clones revealed that both clones secreted IgG1 λ only. LCLs produced 2–5 μg of Ab/ml of conventional culture medium. Genotyping of Patient 1 and 2 infecting viruses was at time of peripheral blood mononuclear cell (PBMC) immortalization and on two times within the past 2 years prior to the immortalization using two different assays. Both assays gave concordant results and indicated that Patient 1 was infected by a genotype 4 isolate while Patient 2 was infected by a genotype 1b isolate. As commercially available HCV genotyping assays may be lacking specificity and to exclude the possibility of dual infection, we further confirmed the above results by the analysis of PCR-derived sequences mapping within the 5′ noncoding region of the HCV genome. Nucleotide sequences derived from cloned quasispecies was compared to published databases (Bukh *et al.,* 1992), and the results confirmed those obtained with the commercial genotyping assays, i.e., that both patients were infected with a single viral type (data not shown). Figure 1 illustrates the staining of SVE2-infected CV-1 cells observed in the IFA using the patients' sera (A and B) or the purified monoclonal antibodies (C and D). The reactivity was localized in the cytoplasm with a predominant perinuclear distribution.

Immunological characteristics of the HMAbs

Western blot analysis, immunofluorescence, and epitope mapping studies. Different approaches were used to characterize the immune reactivity of the produced antibodies. Western blot analysis using denaturing conditions and protein preparations containing subtype 1a- or 1b-derived E2 proteins (Nakano *et al.,* 1997) were performed using the original patients' sera, supernatants from the LCLs, and purified antibodies. While sera of both HCV-infected patients reacted with the E2 1a- and 1b-derived proteins, none of the culture supernatants or purified HMAbs gave a positive signal even when tested at concentrations as high as 10 μg/ml (data not shown). No reactivity could be observed either when culture supernatants or purified antibodies were tested in a peptide scanning ELISA using a panel of synthetic peptides covering a E2 1a sequence (data not shown).

As the above observations suggested that the recognized determinants may be of nonlinear nature, immune reactivity of the different samples was analyzed in IF assays. Cells were transfected with a panoply of plasmids expressing different domains of E2 (see Fig. 2) to try and identify restricted determinant sequences. Results of the IF studies are summarized in Table 2. Serum of Patient 1 recognized multiple determinants mapping within the sequences expressed from various E2-expressing plasmids. In contrast, the HMAb 503 derived from this patient recognized only the near full-length expressed form of E2 (encoded by plasmid pCIE2t) but none of the smaller expressed forms of the antigen. For Patient 2, the serum as well as the derived 108 antibody reacted only against the largest expressed form of E2.

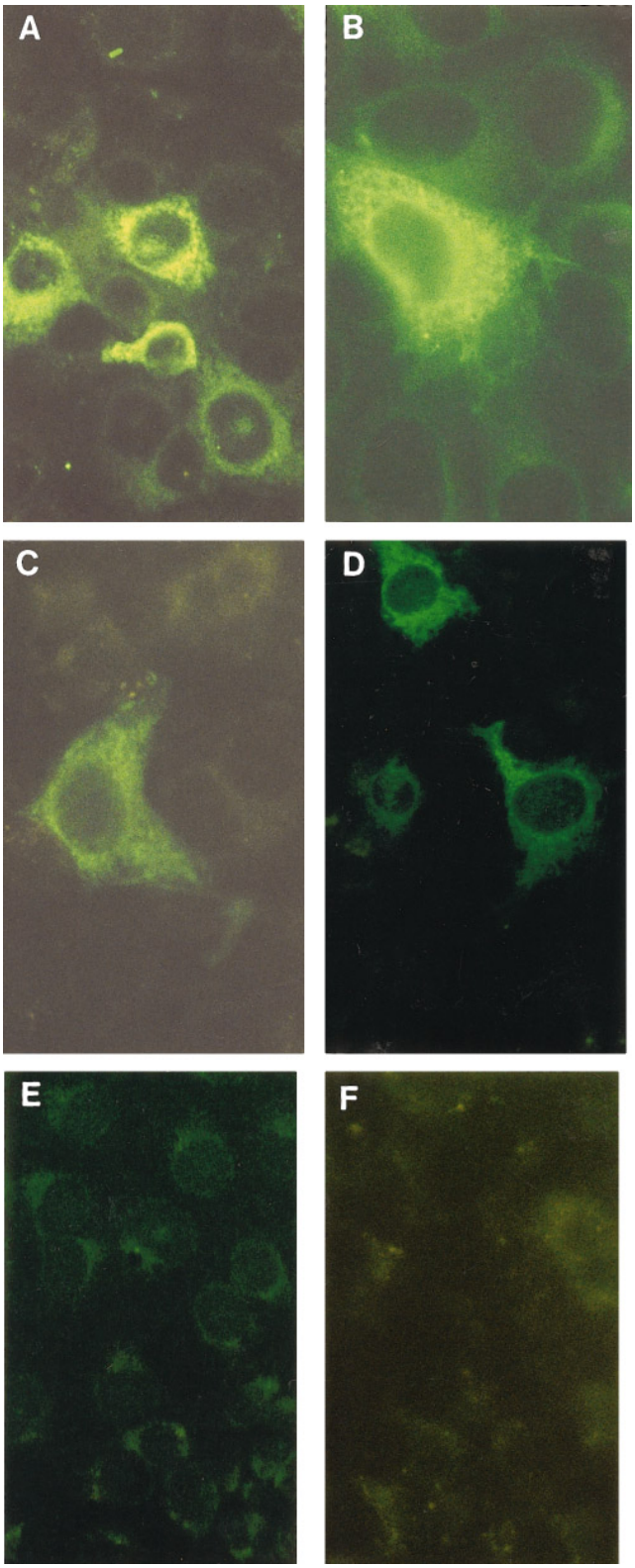

FIG. 1. Indirect immunofluorescence analysis. CV-1 cells were infected with the SVE2 recombinant virus as previously described (Fournillier-Jacob *et al.,* 1996) and immunofluorescence analysis performed using patients's sera (1:20 dilution) or supernatants from the HMAbs producing cell lines. Staining was performed using goat-anti human IgG immune serum coupled with fluoresceine. (A) serum from Patient 1; (B) serum from Patient 2; (C) HMAb 503; (D) HMAb 108; (E) serum from a patient with chronic hepatitis unrelated to HCV. An additional negative control (F) included CV-1 cells transfected with a SVE1 recombinant virus (expressing E1) and stained with the HMAb 503 or 108 (result is shown for HMAb 503 only).

Thus using this approach, we were unable to identify restricted determinant sequences recognized by either of the purified antibodies.

All the above experiments involved a subtype 1a-derived antigen. We evaluated the capacity of the purified monoclonal antibodies to, in addition, recognize a subtype 1b-derived E2 as a way to evaluate their cross-reactive potential. Reactivity of the HMAbs was tested by immunoprecipitation using cells infected with a recombinant Sindbis virus, Sinrep/HCV-BK1–1207, expressing such an antigen. Both antibodies were capable of recognizing the antigen as shown by the observation of strong, specific signals (data not shown).

Taken together with the above results, these data suggest that the HMAbs are capable of recognizing determinants specific of at least two different E2 subtype (1a and 1b)-derived antigens. In addition, they strongly suggest that the antibodies are likely to recognize conformation-dependent determinants.

Immunoprecipitation studies. The absence of reactivity of the HMAbs in Western blotting and by IFA on LTK cells transfected by a panel of vectors expressing different truncated parts of E2 suggests that the Abs recognize conformation-dependent epitope(s). We therefore further evaluated the recognition of E2 by the HMAbs in pulse chase experiments. In addition, as previous reports have suggested that E1 and E2 interact to form complexes which have been proposed to be functional subunits incorporated in the virion particles (Deleersnyder *et al.,* 1997), we also evaluated the ability of the HMAbs to recognize such complexes. These E1E2 complexes are noncovalently associated or stabilized by intramolecular disulfide bonds forming E1E2 aggregates. Covalently associated E1E2 complexes have also been reported that are not believed to be part of the functional subunits of the viral particles (Grakaoui *et al*., 1993; Ralston *et al*., 1993; Dubuisson *et al*., 1994).

The HMAbs recognize an early folded domain of E2. Immunoprecipitations were performed to characterize the proteins recognized by these HMAbs (Fig. 3). Murin anti-E2 MAbs directed at conformation-independent (MAb A11) or conformation-dependent (MAb H2) epitopes were used for comparison (Dubuisson *et al*., 1994; Deleersnyder *et al*., 1997). Under reducing conditions, HMAbs 108 and 503 did not precipitate the E2 protein during the pulse, but after 30 min of chase, a band corresponding to E2 started to be detected with an increased intensity after 60 min (Fig. 3, reducing HMAbs 108 and 503). This is in contrast with results obtained using the murin MAb A11 directed at a conformation-

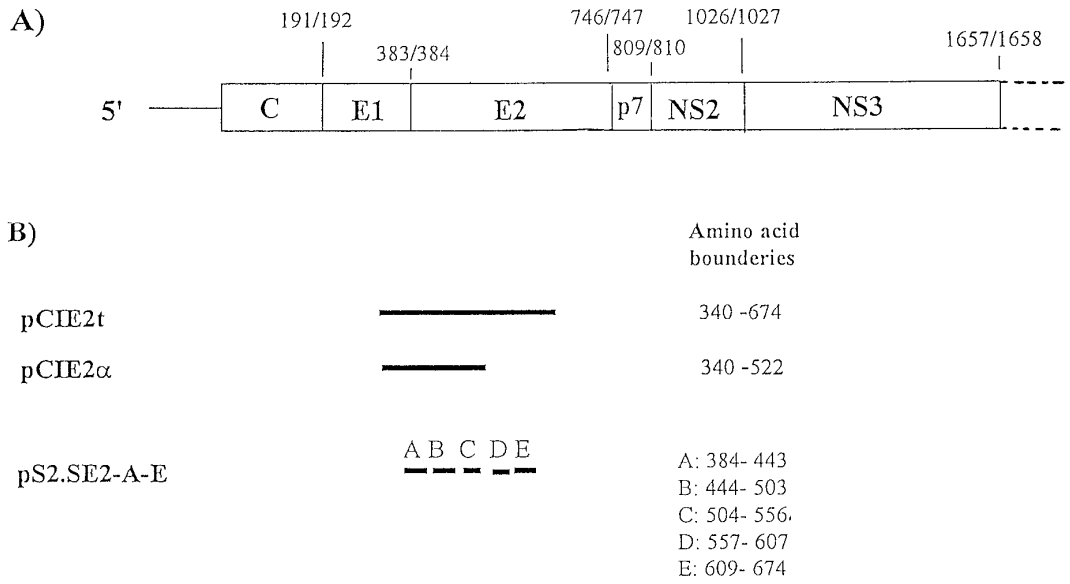

FIG. 2. Plasmids used in the epitope mapping studies. (A) Representation of the HCV genomic domain coding for the viral nucleocapsid (C), the glycoproteins E1 and E2, and the nonstructural proteins p7, NS2, and NS3 (Rice *et al.,* 1996). Amino acid position of the proteolytic cleavage sites are indicated. (B) Map position and amino acid boundaries of the sequences encoded by the different expression plasmids.

independent epitope (Fig. 3, reducing MAb A11). As previously observed with this latter antibody, heterogeneous E2-related products were detected during the pulse probably as a consequence of a translational pause during the synthesis of the NS2 region in the E2-NS2 precursor (Dubuisson and Rice, 1996). For the MAb A11, the intensity of the E2-NS2 precursor precipitated was very high after 30 min of chase and decreased with time, whereas it was low and rather constant for the E2-NS2 protein precipitated by the HMAbs 108 and 503, indicating that E2 was mainly precipitated by the Abs after its cleavage from the E2-NS2 precursor. Comparison with immunoprecipitation performed with the conformation-dependent MAb H2 indicated a greater delay in the detection of E2 (Fig. 3, reducing, MAb H2). These observations indicate that indeed the HMAbs recognize a conformation-dependent domain of E2 which appears early during the maturation process of the E2 protein. The estimated half-time of epitope formation for both HMAbs was around 15 min (data not shown).

The HMAbs can precipitate noncovalent E1E2 complexes. Additional pulse chase experiments were performed under nonreducing conditions and compared with those performed under reducing conditions (Fig. 3). While under reducing conditions, both HMAbs coprecipitated E1, indicating that they recognize E1 and E2 complexes; when immunoprecipitations were performed under nonreducing conditions which prevent the disulfide bonds stabilizing E1E2 complexes, slow migrating bands were also detected on the top of the gels. The latter

TABLE 2

Immune Reactivity of Patients's Sera and of Purified Monoclonal Antibodies (HMAbs) against Truncated Domains of E2

HCV constructs[a]	A	B	C	D	E	pCIE2α	pCIE2t	pcDNA3
Patient 1[b]								
serum	+	−	+	−	−	+	+	−
HMAb 503	−	−	−	−	−	−	+	−
Patient 2[b]								
serum	−	−	−	−	−	−	+	−
HMAb 108	−	−	−	−	−	−	+	−
Mouse polyclonal Ab[c]	+	+	+	+	+	+	+	−

[a] LTK cells were transiently transfected with the indicated plasmids and IFA performed 48 hrs later as described in Major *et al.,* (Major *et al.,* 1995). The pS2.SE2A-E plasmids are according to Nakano *et al.,* (Nakano *et al.,* 1997). The pcDNA3 plasmid (Promega) was used as a negative control.

[b] Patients' sera were tested at 1/20 dilution; supernatants of LCLs or purified HMAbs were used at a concentration up to 10 μg/ml in at least two independently performed experiments.

[c] The efficiency of transfection and proper expression of the plasmids was evaluated in all cases using a reactive hyperimmune serum obtained from mice immunized by direct injection of the plasmid pCIE2t (Nakano *et al.,* 1997).

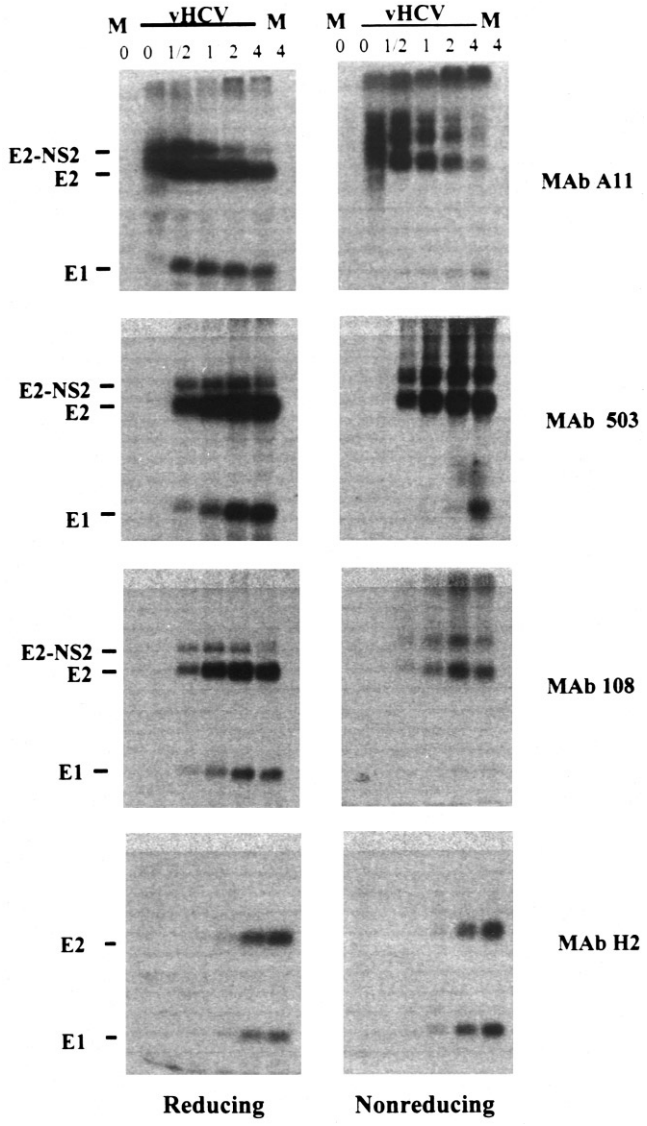

FIG. 3. Immunoprecipitation of E2 and coprecipitation of E1 and analysis of epitope formation under under reducing and nonreducing conditions. Cells coinfected with vTF7–3 and vHCV1–1488 (vHCV) or with TF7–3 alone (M) were pulse labeled for 5 min and chased for the indicated times (in hours). The E2 glycoprotein was immunoprecipitated with HMAbs 108, 503, and mouse MAbs H2 (Deleersnyder *et al.,* 1997) and A11 (Dubuisson *et al.,* 1994). Immunoprecipitates were analyzed under reducing or nonreducing conditions by SDS-PAGE (10% acrylamide). Expected position of HCV-specific proteins are indicated at the left of the figures.

observation suggests that the E1E2 complexes precipitated consisted of noncovalently associated heterodimers and heterogeneous linked aggregates. As previously observed, for MAb H2 which has been, shown capable to recognize a native form of E2, only bands corresponding to E1 and E2 were detected on the gel in that case (Fig. 3, nonreducing, H2, Deleersnyder *et al*., 1997). Under nonreducing conditions, the coprecipitation of the E1 monomeric form with HMAb 108 was poorer with a specific band detected only after a long exposure time as compared with HMAb 503. Thus both HMAbs recognize domain(s) of the E2 protein that appear folded early and would stay accessible as the protein adopts its final conformation as suggested by the coprecipitation of noncovalently associated E1E2 complexes.

All together, our data indicate that both HMAbs 108 and 503 recognize a conformation-dependent determinant (or determinants) and could precipitate E1 and E2 noncovalently associated complexes which are believed to exist on the virion particle.

Neutralization of E2 binding onto cells

The assay recently developed by Rosa *et al.* (1996) allows us to evaluate, in a quantitative fashion, the ability of candidate antibodies to neutralize the binding of highly purified E2 (neutralizing of binding or NOB) onto cells susceptible to HCV infection. Both HMAbs were evaluated in this assay. Percent neutralization obtained at different concentrations of the antibodies are shown in Fig. 4. Results indicate that the HMAb 503 displayed NOB activity and that 50% neutralization of binding was achieved at a concentration of 0.03 μg/ml. No NOB activity could be detected for HMAb 108 at any of the concentration tested. Thus one HMAb purified in our study was capable of NOB activity. This is the first such antibody described to date. Interestingly, the fact that the producing clone (503) was derived from a genotype 4-infected patient while the assay used a genotype 1(a)-derived antigen confirms the cross-reactive potential of this antibody. The data also suggest that antibodies with NOB-activity seem to be targeted at determinants conserved between different viral genotypes.

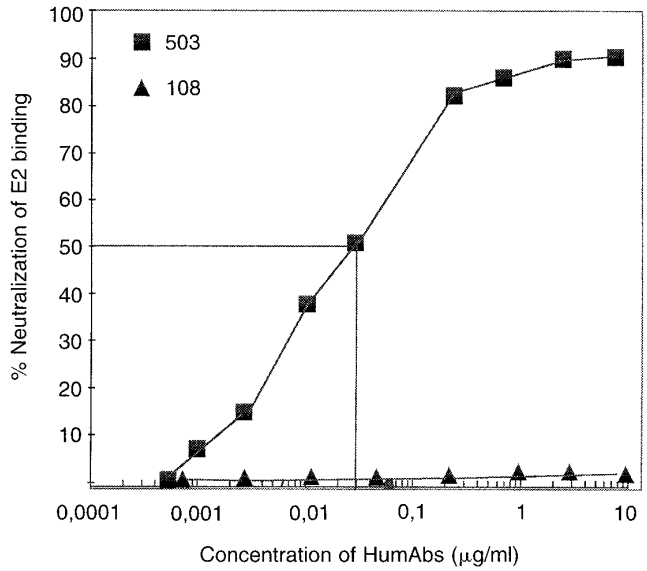

FIG. 4. Percent neutralization of E2 binding by HCV-E2 HMAbs. Various concentrations of anti-E2 HMAbs 503 and 108 were tested for their ability to neutralize binding of purified CHO-expressed E2 protein onto MOLT-4 cells. Neutralization was calculated as described (Rosa *et al.,* 1996) and 50% neutralization titers are indicated.

Competition experiments were performed to determine whether the two antibodies bind to similar or topographically distinct epitopes. The HMAb 108 did not prevent (i.e., did not compete) the detection of the neutralizing activity of the 503 Ab even when it was used at the highest concentration (2.5 μg/ml, data not shown). These results strongly suggest that the HMAbs 108 and 503 recognize different epitopes on the E2 protein.

DISCUSSION

Probing for the B-cell repertoire in viral infections is critical for the understanding of pathogenesis associated with these infections. Human monoclonal antibodies provide an alternative method to do so. Isolation and characterization of such antibodies have been reported in the case of HCV for only a limited number of viral antigens. These include the nucleocapsid, the NS3 and NS4 proteins (Cerino and Mondelli, 1991; Akatsuka *et al.,* 1993; Cerino *et al.,* 1993; Mondelli *et al.,* 1994; Chan *et al.,* 1996) and more recently the glycoprotein E2 (Chan *et al.,* 1996). In the latter case, authors used the phage display technology coupled with the use of synthetic peptides for the screening of the anti-E2 immune reactivity and were able to obtain specific IgG single-chain Fvs that recognized the E2 sequence. While a specific linear epitope sequence was identified, no biological activity for the anti-E2 antibody was described, and the putative role of this antibody in the control or progression of infection remains undefined.

We have focused the present study on the glycoprotein E2 of HCV and selected a screening assay that specifically allows the detection of anti-E2 antibodies capable of recognizing E2 directly expressed in cells without the requirement of antigen purification. Such a screening assay was chosen to enhance the chances of purifying antibodies directed at conformation-dependent determinants. The assay was also based on expression of a genotype 1a-derived antigen, thus allowing for the characterization of cross-reactive anti-E2 antibodies and epitopes. Such reagents could be particularly useful for the development of therapeutic or preventive strategies to fight infection by a highly mutable agent such as HCV. Using this approach, we have successfully derived two clones producing anti-E2 antibodies from two HCV chronically infected patients. The first clone (clone 503) was obtained from one patient (Patient 1) infected by a genotype 4 isolate while the second clone (clone 108) was derived from a second patient (Patient 2) infected by a genotype 1b isolate. We showed that the HMAbs displayed, in addition, a good reactivity against a genotype 1b antigen, suggesting that the determinant(s) targeted by these antibodies is conserved among at least two of the main prevalent viral subtypes found in the world (subtypes 1a and 1b).

Our results indicate that the determinants recognized by the HMAbs produced in our study are targeted at conformation-dependent domains of E2. Indeed, we failed to identify linear determinants using different screening approaches, including peptide-scanning, Western blots, and immunofluorescence analysis using expressed truncated domains of the protein. On the other hand, immunoprecipitation studies performed under reducing or nonreducing conditions indicated that the HMAbs recognized a conformation dependent-determinant. Under nonreducing conditions, we showed that these antibodies precipitated covalently as well as noncovalently associated E1E2 complexes. The latter are thought to be functional subunits incorporated in the virion particle (Deleersnyder *et al.,* 1997). The present data, in particular obtained from kinetic analysis of epitope formation strongly indicate that the two HMAbs may recognize domains of the E2 protein that appear to be folded early. Such domains would stay accessible as the protein further matures, until it adopts its final conformation characteristic of the form of E2 susceptible to be present on the surface of virions. The kinetic analysis, together with the NOB data (i.e., one antibody, 503, displaying NOB activity, the other one, 108, displaying no NOB activity) also suggest that the two antibodies recognize different determinants. Alternatively, that affinity of the antibodies for the E2 protein differs. Studies are ongoing to try and identify such determinants.

The most encouraging result of our study was the demonstration that one of the HMAbs displayed strong NOB activity. Our observations together with those of Rosa *et al.* (1996) indicate that the determinant(s) recognized by NOB antibodies are likely directed at conformation-dependent domains of E2, domains that appear to be conserved between different genotypes. Such domains seem to be distinct from the hypervariable region 1 (HVR) that has been shown to contain neutralization epitopes. In a recent study, Zibert *et al.* (1997) have been able to correlate early appearance of antibodies directed at a nonconformational structure found in the HVR with acute self-limited infection. Results from the study suggests the critical existence and role of antibodies directed at a linear determinant of E2 in the control of HCV infection, observations that are in agreement with a study originally performed in the chimpanzee model by Farci *et al.* (1996). Authors in this latter study generated a hyperimmune serum directed at a peptide from the HVR, a serum that contained antibodies capable of neutralizing the infectivity of a well-characterized inoculum *in vitro*. A similar experiment was also performed by Shimizu *et al.* (1996). Thus all of these studies strongly suggest that neutralization of HCV would mostly be type-specific, involving the participation of variable, nonconserved epitopes. Nonetheless, recent observations have begun to suggest the existence of other neutralization determinants, cross-reactive and not directed at the HVR. In the vaccination study by Choo *et al.* (1994), induced

neutralizing antibodies were not directed at the HVR of E2 but apparently at other determinants carried by the antigen. Abrignani has recently observed a correlation between spontaneous resolution of chronic infection and appearance of high anti-NOB antibody titers (Abrignani 1997). In patients described in this study, high or measurable neutralization of binding of E2 was not restricted to sera from patients infected with genotype 1a isolates, thus suggesting the existence of cross-reactive epitopes such as those described in our study. While it is difficult to find a direct correlation between NOB titers of a purified MAb and titers found in patients' sera (both patients in our study had similar NOB serum titers ranging between 1:5000 and 1:6000), we can stress the fact that antibody 503 has an NOB activity detectable at very low concentration (0.03 μg/ml), suggesting a potent activity.

The HMAbs produced in our study should be useful tools to study further the biogenesis, folding, and assembly of HCV glycoproteins as well as for characterization of the virion structure and a putative cell-surface receptor. As one of them (Ab 503) represents the first HMAb described to date as having NOB activity, it would be also of evident interest to determine the performances of this antibody in passive immunization studies. Antibody infusion studies have demonstrated, in the case of lentiviruses, a beneficial role of administered neutralizing antibodies in the control and even the prevention of infection in different animal models (Putkonen *et al.,* 1991; Emini *et al.,* 1992; Conley *et al.,* 1996).

MATERIALS AND METHODS

Patients

Two patients were enrolled in the study. HCV infection was determined by the RIBA III assay (Abbott Laboratories). At time of PBMC immortalization, both patients had chronic hepatitis as determined by histological examination and positive PCR assays. In one of these two patients, Patient 1, despite detectable HCV RNA by PCR in the serum, serum ALT (alanine amino transferase) levels were and remained normal (mild hepatitis). In contrast, ALT levels remained persistently elevated in Patient 2, and infection in this patient was characterized by cirrhosis.

Screening assay for the detection of anti-E2 antibodies

Detection of anti-E2 antibodies has been reported to be tightly dependent on the antigen production method (Chien *et al.*, 1993; Hsu *et al.,* 1993; Lesniewski *et al.*, 1995). Eukaryotic but not prokaryotic expression of HCV E2 has been shown to allow for proper processing and glycosylation of the protein (Selby *et al.,* 1993). In our study, we used as screening assay for anti-E2 antibodies an eukaryotic-expressed E2 antigen analyzed under a native form, i.e., visualized by an immunofluorescence assay (IFA). Such a detection assay has been previously used by Fournillier-Jacob *et al.* (1996b) and shown to be particularly efficient for antibody detection. Briefly, a recombinant plasmid, pCW18 E2, expressing HCV E2 amino acid sequence 371–746 from the prototype strain H (genotype 1a) was used to transfect CV-1 cells together with a helper SV40 mutant virus to generate the stock of recombinant virus expressing E2 (SVE2, Wychowski *et al.,* 1986; Fournillier-Jacob *et al.,* 1996a). SVE2 virus was used to further infect CV-1 cells, and immunofluorescence analysis were performed using sera from infected patients and supernatants from EBV-immortalized B cells as previously described (Fournillier-Jacob *et al.,* 1996b). Cells were fixed in methanol:acetone (3:7) prior to analysis.

Generation of HMAbs producing cells lines

Generation of HMAbs producing cell lines was performed as previously described (Seigneurin *et al.,* 1983; Desgranges *et al.,* 1988; Boyer *et al.,* 1991). Briefly, after Ficoll isolation, PBMCs were exposed to EBV culture supernatant (1 ml of B95.8 strain supernatant with a titer of 10^{-3} TD^{50}/ml for 5×10^6 PBMCs) at room temperature. After incubation, they were diluted in medium at concentration ranging from 50 to 100×10^3 cells per well. After 2–4 weeks, the supernatants were screened for anti-E2 reactivity by the SVE2 CV-1 IFA. LCLs were further subcloned twice at 2–20 cells per well with 50×10^3 irradiated (2500 rads) allogenic PBMCs. Two persistently positive clones derived from the two patients were obtained.

Determination of viral load and genotype

Serum viral loads were determined using the bDNA assay version 2.0 (quantiplex HCV RNA Assay, Chiron Diagnostics). HCV genotypes were determined using two different methods. The first method was based on the detection of genotype-specific antibodies directed at the nonstructural antigen 4 (NS4) and was determined using the MUREX 1–6 serotyping assay according to the manufacturer's instruction (MUREX Diagnostics SA, Bhattacherjee *et al.,* 1995). The second one was based on the amplification of viral sequences from the 5′ noncoding region (NCR) of the genome using genotype/subtype specific primers and was performed using the INNO-LIPA assay (Innogenetics S.A.).

Typing, purification, and determination of the concentration of antibodies produced by the two clones

Supernatants from each clone were tested by IFA on CV-1 cells infected with the recombinant SVE2 virus, and staining was revealed using specific secondary antibodies for human IgM, IgG or IgA (Byosis), IgG1, IgG2, IgG3, and IgG4 subclasses (Sigma Immuno Chemical Co.) and

for λ and κ light chains (Dakopatts). A protein A (Pharmacia) column was used for affinity purification of supernatant producing HMAbs. The determination of antibody concentration in culture supernatants was performed by ELISA as previously described (Boyer *et al.,* 1991).

Western blot analysis and peptide scanning

For Western blotting analysis, baculovirus-expressed E2 proteins from a genotype 1a and 1b sequence were used as previously described (Nakano *et al.,* 1997). Patients' sera (1:50), supernatants from the two clones, and purified HMAbs (tested at a concentration as high as 10 μg/ml) were used. Epitope mapping using patient's sera as well as purified antibodies was performed using a panoply of synthetic peptides covering the entire E2 open reading frame as previously described (courtesy of A. M. Prince, Wang *et al.,* 1996). The synthetic peptides were mostly 12-mer with 6-aa overlap between successive peptides, corresponding to the sequences of HCV-H strain (genotype 1a) E2 protein. There were a total of 57 peptides for E2 (aa 384–727), all of which were synthesized by AnaSpec.

Epitope mapping

The two HMAbs obtained were evaluated for reactivity by IFA on LTK cells transfected by a panoply of vectors expressing truncated domains of the E2 protein (see Fig. 2). E2 sequences were cloned directly under the CMV promoter of the pcDNA3 plasmid (Promega) for plasmids pCIE2α and pCIE2t or expressed as fusion proteins with the hepatitis B virus surface antigens for plasmids pS2S.E2A-E using standard techniques and as previously described (Sambrook *et al.,* 1989; Nakano *et al.,* 1997). All DNA preparations were generated using Qiagen purification columns (Qiagen) according to the manufacturer's instructions. LTK cells were transfected using 10 μg of DNA in presence of Lipofectamine (Gibco BRL). The immune-reactivity of cell supernatants and of purified HMAbs were tested by IFA at 48 h postinfection as previously described (Major *et al.,* 1995). Positive control included the use of a reactive hyperimmune serum generated from mice immunized by the direct injection of the plasmid pCIE2t (Nakano *et al.,* 1997). Negative controls included the use of uninfected LTK cells as well as CV-1 cells infected with a recombinant SV40 virus expressing E1 (Fournillier-Jacob *et al.,* 1996a).

Metabolic labeling and immunoprecipitation

For the purpose of our study, different recombinant viruses were used. These included (1) a recombinant vaccinia virus vTF7.3 expressing the T7 DNA-dependent RNA polymerase (Fuerst *et al.,* 1986), (2) a panoply of recombinant vaccinia viruses expressing HCV-H amino acid sequences, vHCV 170–809, vHCV 371–809, vHCV 1–1488, and vHCV 370–661 (Grakoui *et al.,* 1993; Major *et al.,* 1995; Fournillier-Jacob *et al.,* 1996a; Michalak *et al.,* 1997), and (3) a recombinant Sindbis virus (Sinrep/HCV-BK1–1207) expressing the structural proteins of a genotype 1b strain, the BK strain (Dubuisson *et al.,* 1994). Viral stocks were generated in CV-1 monolayers (for the vaccinia viruses) or in BHK-21 cells (for the Sindbis virus) as described (Bredenbdeeck *et al*., 1993; Dubuisson *et al.,* 1994). Cells were infected and metabolically labeled with ^{35}S-translabel (ICN) as previously described (Dubuisson *et al.,* 1994; Dubuisson and Rice, 1996). Cells were lysed with 0.5% NP-40 in 10 mM Tris-HCl (ph 7.5), 150 mM NaCl, and 2 mM EDTA Iodoacetamide (20 mM) was included in the lysis buffer for experiments in which disulfide bond formation was assayed. Immunoprecipitations were carried out as described (Dubuisson *et al.,* 1994; Dubuisson and Rice, 1996). For quantitative experiments, autoradiographs were analyzed by densitometry.

Neutralization assay

We assessed the ability of the HMAbs to neutralize the binding (NOB) of E2 to MOLT 4 cells in the assay recently developed by Rosa *et al.* (1996). The assay was run in 96 U-bottom microplates. Briefly, 20 μl of recombinant CHO $E2_{384–715}$ proteins at 0.5 μg/ml was mixed with various dilution of anti-E2 HMAbs and control HMAbs (Boyer *et al.,* 1991; Rosa *et al.,* 1996). After incubation at 4°C for 1 h, the mixture was added to MOLT-4 cells (10^5 cells per well). After washing, cells were subsequently incubated with 1/100 dilution of human serum with anti-E2 immunoglobulins which recognizes E2 bound to target cells. Cells were washed and incubated with fluoresceine isothiocianate-conjugated antiserum to IgG. Fluorescence was analyzed with a FACScan flow cytometer. Specific neutralization was calculated as follow: [(positive control MFI − experimental MFI)/(positive control MFI − negative control MFI)] × 100 where (MFI) = mean fluorescence intensity of the cell population which directly relates to the surface density of fluorescently labeled HCV proteins bound to the cells. MFI values of cells incubated with or without HCV proteins and with the HCV HMAbs or HCV-negative HMAbs or pre-immune sera (Rosa *et al.,* 1996) are compared. The threshold of positivity is set for each experiment by flow cytometric analysis of cells without HCV proteins bound that have been incubated with antisera to HCV proteins and the fluorescein isothiocyanate-labeled second antibody. For competition binding analysis, antibodies were biotinylated as followed: 1 mg/ml of the antibodies in 0.4 M phosphate buffer were incubated with N-N-dimethylformamide biotin at 2 mg/ml at 4°C for 2 h and dialyzed extensively against PBS overnight. Suboptimal concentration of biotinylated HCV E2 1a was preincubated with different amounts of unlabelled HMAbs 108 and 503 (20 min at 37°C); then, on ice were added 3×10^4 cells/well. After 30 min, samples were washed twice and streptavidin

peroxydase conjugate was added; after 20 min on ice, samples were read.

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